Bone regeneration using a synthetic matrix containing enamel matrix derivate

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Abstract: PURPOSE: The aim of the present study was to test whether the delivery of enamel matrix derivate (EMD) via synthetic polyethylene glycol (PEG)-based hydrogels with and without RGD sequences enhances bone formation in vivo. MATERIAL AND METHODS: In each of 10 rabbits, four titanium cylinders were placed on the external cortical bones of their calvaria. The following four treatment modalities were randomly allocated: One of the four cylinders was left empty (control), the other three were filled with a combination of PEG matrix with hydroxyapatite/tricalciumphosphate (HA/TCP) granules and EMD in a concentration of 100 μg/ml (test 1) or 500 μg/ml (test 2) or 500 μg/ml and RGD peptide (test 3). After 8 weeks, the animals were sacrificed and ground sections were obtained for histological analysis. For statistical analysis, the Kruskal-Wallis test was applied (P<0.05). RESULTS: The histomorphometric analysis revealed a statistically larger area fraction of newly formed bone in the EMD 500/RGD group (54.8±14.5%) compared with the control group (28.7±10.3%) and the EMD 500 group (31.2±14.1%) and non-significantly higher area fraction compared with the EMD 100 group (38.2±10.4%). The percentage of mineralized bone showed no statistically significant differences among the four groups. The mean percentage of mineralized bone was 13.6±3.3% in the control group, 14.2±5.8% in the EMD 100 group, 11.69±5.9% in the EMD 500 group and 15.66±5.2% in the EMD 500/RGD group. No statistically significant difference regarding the bone-to-graft contact between the EMD 100 group (23±15.7%), the EMD 500 group (22.2±14.6%) and the EMD 500/RGD group (21.6±8.8%) was observed. CONCLUSIONS: The combination of a PEG matrix containing EMD with HA/TCP granules had no effect on the formation of mineralized bone tissue in rabbit calvaria. The addition of RGD peptide to the PEG/EMD 500 combination increased the area fraction of newly formed bone compared with the other treatment groups. Further studies are indicated to study a possible synergistic effect of EMD and RGD. © 2011 John Wiley Sons A/S.

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BONE REGENERATION USING A SYNTHETIC MATRIX CONTAINING ENAMEL MATRIX DERIVATE

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Keywords

enamel matrix protein, EMD, polyethylene glycols, RGD, bone regeneration, graft material, animal study, bioactive factor

Abstract

Purpose: The aim of the present study was to test whether the delivery of Enamel matrix derivate (EMD) via synthetic Polyethylene glycol (PEG) -based hydrogels with and without RGD sequences enhances bone formation in vivo. **Material and methods**: In each of 10 rabbits 4 titanium cylinders were placed on the external cortical bones of their calvaria. The following 4 treatment modalities were randomly allocated: One of the 4 cylinders was left empty (control), the other 3 were filled with a combination of PEG matrix with Hydroxyapatite/Tricalciumphosphate (HA/TCP) granules and EMD in a concentration of 100µg/ml (Test 1) or 500µg/ml (Test 2) or 500µg/ml and RGD peptide (Test 3). After 8 weeks the animals were sacrificed and ground sections were obtained for histological analysis. For statistical analysis the Kruskan-Wallis test was applied ($p<0.05$). **Results**: The histomorphometric analysis revealed a statistically larger area fraction of newly formed bone in the EMD 500/RGD group (54.8 ± 14.5%) compared to the control group (28.7 ± 10.3%) and the EMD 500 group (31.2 ± 14.1%) and non-significantly higher area fraction compared to the EMD 100 group (38.2 ± 10.4%). The percentage of mineralized bone showed no statistically significant differences among the four groups. The mean percentage of mineralized bone was 13.6 ± 3.3% in the control group, 14.2 ± 5.8% in the EMD 100 group, 11.69 ± 5.9% in the EMD 500 group and 15.66 ± 5.2% in the EMD 500/RGD group. No statistically significant difference regarding the bone to graft contact between the EMD 100 group (23.0 ± 15.7%), the EMD 500 group (22.2 ± 14.6%) and the EMD 500/RGD group (21.6 ± 8.8%) was observed. **Conclusions**: The combination of a PEG matrix containing EMD with HA/TCP granules had no effect on the formation of mineralized bone tissue in rabbit calvaria. The addition of RGD peptide to the PEG/EMD500 combination increased the area fraction of newly formed bone compared to the other treatment groups. Further studies are indicated to study a possible synergistic effect of EMD and RGD.

Introduction

Tooth extraction is followed by a remodeling processes of the alveolar ridge resulting in partial loss of the buccal bone contour (Araujo & Lindhe 2005). A large variety of methods and materials for the regeneration of lost alveolar bone have been described (Hammerle & Karring 1998). Although autologous bone is still considered to be the “gold standard”, the limited availability and the donor site morbidity have led to a shift towards the use of xenogenic or allogenic materials (Nkenke et al. 2002; Nkenke et al. 2001). Some of these materials are well documented and have proven their efficacy and safety over a long period, but they are associated with long healing periods, since they lack osseoinductive properties (Iezzi et al. 2007; Traini et al. 2007). Grafting materials originating from animal sources can cause difficulties in terms of patient acceptance, immune response and possible transmission of infectious agents that can never be completely excluded. As a fully synthetic material, HA/TCP overcomes these shortcomings and has shown effectiveness as a bone substitute for guided bone regeneration (GBR) in several studies (Artzi et al. 2008; Cordaro et al. 2008; Froum et al. 2001; Schwarz et al. 2007, Zafiropoulos et al. 2007)

The induction of bone formation remains a challenge in tissue regeneration. Numerous preclinical and clinical studies have shown a positive effect of growth factors and differentiation factors in hard and soft tissue regeneration (Cochran et al. 1999; Howell et al. 1997; Jung et al. 2003; Jung et al. 2008; Jung et al. 2005; Lynch et al. 1991). The regenerative potential also depends on the method of application and release kinetics of the bioactive substances from their carrier (Sigurdsson et al. 1996; Hunt et al. 2001).

Enamel matrix derivate (EMD) is an extract from porcine tooth buds. It is composed of several proteins, up to 90% of which are amelogenins. The residual 10% are proline-rich non-amelogenins. Enamel matrix proteins are secreted by ameloblasts during tooth

formation and regulate enamel mineralization (Simmer & Fincham 1995). They are also secreted by epithelial cells of the Hertwig‘s root sheath during root formation and affect the formation of periodontal tissues, primarily acellular cementum (Hammarstrom 1997). Therefore, EMD has been mainly used in periodontal regenerative treatment, especially for furcation and intrabony defects (Donos et al. 2003; Froum et al. 2001; Hoffmann et al. 2006; Jepsen et al. 2004; Meyle et al. 2004; Sanz et al. 2004; Tonetti et al. 2002).

Very little data is available regarding the effect of EMD for periimplant bone regeneration (Casati et al. 2002). As matrix proteins EMD shows very low solubility at physiological pH, but is soluble under acidic conditions. Commercially available EMD (Emdogain®) is dissolved in a slightly acidic propylene glycol alginate (PGA) gel. One problem in applying Emdogain® for periimplant bone regeneration is that its PGA carrier gel is designed to collapse and release the EMD at the tooth root under physiological conditions and thus does not provide space keeping properties or the retention of the protein in a larger volume. The use of an optimized matrix as delivery system for EMD could overcome this problem. The combination of a bone substitute (e.g. HA/TCP) potentially provides additional stability of the compound helping to keep the substances at the site of bone regeneration.

Polyethylene glycol (PEG) based hydrogels have been shown to be effective delivery systems for bioactive substances since these matrices allow optimal cell ingrowth as well as retention and release of bioactive proteins such as recombinant human bone morphogenetic protein-2 (rhBMP-2) and parathyroid hormone (PTH) (Jung et al. 2008; Jung et al. 2007a; Jung et al. 2007b; Lutolf et al. 2003). The ingrowth of cells into these matrices can be facilitated by the presence of RGD-containing peptides, an amino acid sequence composed of arginine, glycine and aspartate. RGD sequences are mainly found in extracellular matrix proteins (e.g. fibronectin) and mediate cell adhesion, cell migration and signal transduction via binding to integrins, a subgroup of cell surface receptors

(Akiyama 1996). The possible application of EMD with an optimized carrier material with and without RGD has not been investigated yet.

Hence, the aim of the present study is to test if delivery of EMD in different concentrations via synthetic PEG-based hydrogels with and without RGD sequences enhances bone formation in vivo.

Materials and Methods

The present animal investigation was evaluated and approved by the responsible Animal Research Ethics Committee at the University of Zurich, Switzerland.

Synthetic matrix and bioactive peptides

The synthetic matrix used as the carrier in the present study was a polyethylene glycol-based hydrogel (Institut Straumann AG, Basel, Switzerland). This gel was formed by a reaction of a 4-arm PEG with acrylate endgroups with a linear PEG with thiol endgroups in an aqueous buffer system (triethanolamine/HCl) (Elbert et al. 2001). The PEG termini connected through a highly self-selective addition reaction, forming an elastic gel network. Immediately before application, both PEG solutions were sterile filtered and mixed with 0.15 g of HA/TCP granulate in a distribution of 60%:40% and a particle size of 400-700µm (Straumann BoneCeramic, Institut Straumann AG, Basel, Switzerland).

For the activated gels stock solutions of a lyophilized enamel matrix derivate (EMD) and a 9 amino acid cys-RGD peptide (Bachem, Bubendorf, Switzerland) in dilute (0.10%) acetic acid were added first to the PEG-acrylate solution, resulting in the formation of covalent bonds between the cystein-residues and the PEG-acrylate upon gelation, which was started by adding the triethanolamine buffer solution. The final concentrations for the peptides were 350 µg/ml gel for cys-RGD and 100 or 500 µg/ml gel for EMD.

The surgical procedure, the histological preparation, the histomorphometric and statistic evaluation were performed according to a previous animal study (Jung et al. 2007b). In brief:

Surgical procedure

The surgical procedure was performed in 10 adult New Zealand white rabbits. During a standardized surgical procedure 4 evenly distributed circular slits, 6 mm in diameter and 1 mm in depth, were prepared with a trephine bur bilaterally in the parietal and frontal bones. Without removal but after perforation of the external cortical plate inside the created circles with a round bur, specially designed 7x7 mm large cylinders made of c. p. titanium with a machined surface on their inside were screwed into the slits.

One of the 4 cylinders served as a control and was left empty. Another cylinder contained the enamel matrix derivate in a concentration of 100 µg/ml gel (test 1), a third cylinder in a concentration of 500 µg/ml gel (test 2). The fourth cylinder contained EMD in a concentration of 500 µg/ml gel in combination with cys-RGD (test 3). The position of the cylinders in each animal was randomly assigned by rotating the position of the treatment modalities in a clockwise direction. After application of the test substances into the cylinders they were closed with a titanium lid and the skin flap was adapted and sutured for primary healing (Fig.1-3).

Histologic preparation

After 8 weeks the rabbits were sedated with barbiturates and sacrificed by an overdose of Ketamin. The specimens were sectioned in the frontal plane through the middle of the cylinders. 200 µm thick sections were obtained, ground and polished to a uniform thickness of 60-80 µm (Donath & Breuner 1982). The specimens were surface-stained with toluidine blue (Schenk et al. 1984). One central section was selected to perform histomorphometric measurements.

Histomorphometry

The mineralized bone tissue content was assessed by applying standard morphometrical techniques (Gundersen et al. 1988; Weibel 1980). Measurements were carried out in a light microscope at a magnification of 160x, using an optically superimposed eyepiece test grid composed of 100 points and 10 cycloid lines (Schenk & Olah 1980). The graft to bone contact was calculated by the number of intersections between graft particles and the outlines of either mineralized bone or non-mineralized tissue.

In order to respect not only the amount of newly formed bone but also its extension and distribution within the cylinders a quantitative evaluation of the area of bone regeneration within the cylinders was performed using a pixel count of histological digital images in an image analysis program (Adobe® Photoshop® 7.0.1) (Area of bone regeneration [%] = pixel number of the bone area X 100/ total pixel number of the cylinder) (Jung et al. 2008).

Statistical analysis

Mean values and standard deviations were calculated based on point measurements or cycloid measurements. The primary unit for statistical analysis was the cylinder. All values are displayed including the median. Significant differences were identified by a Kruskan-Wallis test and confirmed between groups by a Mann-Whitney U test.. Statistical significance was set at $P<0.05$. The statistical analysis was performed by using a statistical software package (SPSS 16 for Windows).

Results

During the experiment, all animals showed an uneventful healing in the area of surgery. No reductions in body weights were noted, and no postoperative infections were observed. Upon specimen retrieval 33 cylinders were found to be stable and in the same

position as at placement. However, 7 out of 40 cylinders were loose and therefore excluded from further analysis (2 in the control group, 2 in test 1, 2 in test 2 and 1 in the test 3 group)

Descriptive histology

The qualitative evaluation of the histological specimens showed variable amounts and patterns of bone formation in the 4 treatment groups. The empty cylinders (control group) presented a structure with few trabeculae and large marrow spaces. Adjacent to the inner walls of these cylinders the bone trabeculae were oriented in a parallel manner. The bone formation was limited to the lower third of the cylinders (Fig. 4).

In cylinders containing the PEG matrix with EMD in a concentration of 100µg/g gel (test 1 group) the amount of newly formed bone varied greatly. Similar to the control group the trabeculae neighboring the inner walls were aligned parallel to the titanium surface. The extension of the newly formed bone varied between the lower to the upper third of the cylinders (Fig. 5).

A similar situation was found in the cylinders filled with PEG matrix with EMD in a concentration of 500µg/g gel (test 2 group). A large variety of new bone was observed (Fig. 6).

The highest amount of newly formed bone was observed in the group containing EMD 500µg/g combined with RGD (test 3 group). In most cases the new bone reached the upper third of the cylinders and was evenly distributed among the lower two thirds, embedding the HA/TCP granules (Fig. 7, 8). All features of lamellar bone structure (osteons, Haversian canals, osteoblasts and -clasts etc.) could be observed.

Histomorphometry

The quantitative histomorphometric analysis revealed that the content of mineralized bone tissue was very similar in all the groups, (Fig. 9). Therefore, no significant difference was detectable between the groups (Table 1). The difference between the means was highest between EMD 500 (11.7 ± 5.6%) and EMD 500/RGD (15.7 ± 5.2%). On the other hand, the quantitative evaluation of the area of bone regeneration revealed a statistically significant larger bone area in the EMD 500/RGD (test 3) group compared to the control group (Fig. 10; Table 2). Among the groups containing EMD, the most extended area of bone formation was found in the EMD 500/RGD (test 3) group (54.8 ± 14.5%) compared to the other EMD groups (test 1: 38.2 ± 10.4%; test 2: 31.2 ± 14.1%). The area of bone formation was significantly increased in the EMD 500 containing groups, when RGD peptides were added.

Regarding the bone to graft contact the EMD 100 group, the EMD 500 group and the EMD 500/RGD group showed no statistically significant difference (23.0 ±15.7%, 22.2 ± 14.6% and 21.6 ± 8.8% respectively) (Fig. 11; Table 3).

Discussion

The results of the present study indicate that the combination of a polyethylene glycol (PEG) matrix containing enamel matrix protein (EMD) with HA/TCP granules has a limited effect on the formation of mineralized bone tissue in the rabbit calvaria. However, the area fraction of bone formation was significantly increased by the addition of RGD peptide to the PEG/EMD500 group without affecting the graft to bone contact.

The topical application of enamel matrix protein is well documented in periodontal therapy and is effective in the regeneration of periodontal tissues including bone. Animal and clinical studies have shown the effectiveness of EMD in the treatment of infrabony (Cochran et al. 2003; Froum et al. 2001; Heijl et al. 1997; Sanz et al. 2004; Sculean et al.

2004; Silvestri et al. 2003; Tonetti et al. 2002) and furcation type defects (Donos et al. 2003; Hoffmann et al. 2006; Jepsen et al. 2004; Meyle et al. 2004), providing superior clinical results than open flap surgery alone and similar results as by applying GTR techniques.

Although it stimulates osteoblasts in vitro (Jiang et al. 2006) EMD has not been tested clinically for regeneration of non-periodontal bone defects. In an animal study, EMD was combined with deproteinized bovine bone mineral (DBBM) in one group and with a propylene glycol alginate (PGA) carrier in another and inserted into muscular tissue, without contact to any bone tissue (Donos et al. 2006). After 2 and 4 months, the DBBM particles were encapsulated, regardless of the presence of PGA and no bone formation was observed indicating that neither EMD alone nor in combination with PGA is able to induce bone formation. A similar study in mice has shown that EMD is not osteoinductive but it can be osteoconductive in certain concentrations in combination with demineralized freeze-dried bone allograft (DFDBA) (Boyan et al. 2000). A comparative study in rabbits with a similar design as the present study showed that EMD had little effect on bone formation in combination with β-TCP compared to β-TCP alone (Murai et al. 2005). Using EMD alone or in combination with DBBM for bone regeneration at the lateral rat ramus, no effect on the formation of new bone under dome-shaped PTFE capsules was observed compared to the use of the capsule alone in another investigation (Donos et al. 2005). These results are in agreement with the present study revealing no effect of EMD in combination with HA/TCP on bone regeneration, even in higher dosages.

In a recent animal model EMD has been compared with rhBMP-2 and DFDBA for the regeneration of critical size calvaria bone defects. It was shown that, without the use of a bone substitute, unlike rhBMP-2, both DFDBA and EMD did not lead to a regeneration of the bone defect (Intini et al. 2008).

EMD has also been investigated for periimplant bone regeneration. An in vitro study demonstrated a concentration-dependent positive effect of EMD on human osteoblast-like cells on a titanium surface (Schwarz et al. 2004). In an animal study with artificial dehiscence type defects it was observed, that the application of EMD resulted in a similar amount of bone formation as GBR with a resorbable membrane (Casati et al. 2002). However, only the combination of EMD and the membrane showed a significantly higher area of bone formation compared to the untreated control group in that study, suggesting that EMD has the potential to stimulate bone formation even in the absence of periodontal ligament cells. The addition of EMD into drill holes before implant placement did not have a beneficial effect on the osseointegration in another animal study (Franke Stenport & Johansson 2003). According to the observation of the growth pattern in the present study, a layer of newly formed bone was found on the inner wall of the titanium cylinders in all groups. A difference in the amount or the pattern of growth along the titanium surface was not found among the groups. The titanium surface seems to be a guiding structure for the cells involved in bone formation independently of the presence or absence of EMD or RGD.

The addition of RGD to the PEG-EMD 500 combination has led to a significant increase in area of bone formation within the titanium cylinders. Several experimental studies have shown an increased bone formation on RGD coated implant surfaces compared to uncoated implants (Elmengaard et al. 2005; Schaffner et al. 1999; Schliephake et al. 2005). Since RGD sequences play an important role in cell adhesion, cell migration and signal transduction (Akiyama 1996; Pierschbacher & Ruoslahti 1984), it can be assumed, that these properties are beneficial for the formation of new bone in connection with the bone substitute HA/TCP. However, although RGD has been suggested to be a mediator

for osteoblast adhesion to Hydroxylapatite (HA) (Okamoto et al. 1998), the bone-to-HA/TCP contact seems not to have been influenced by RGD in the present study. The fact that the bone–to-graft contact is not influenced by the presence of the RGD peptides might indicate their absence from the HA/TCP surface, most likely due to the fact that the cys-RGD peptides were allowed to covalently bind to the PEG hydrogel by their addition to the PEG acrylate solution prior to their first contact with the HA/TCP particles.

According to a recent in vitro study (Lutolf et al. 2003) the ingrowth of osteoblasts into the PEG matrix is facilitated by the presence of RGD. This might explain the finding that the addition of RGD to 500 µg/ml EMD significantly increased the area of newly formed bone. It can be speculated that the presence of RGD within the PEG gel facilitates the ingrowth of osteoblasts and leads to a broader distribution of the bone tissue within the cylinders. Thus, also in this animal model, the presence of RGD peptides in the matrix is beneficial for bone formation.

The same study design and the same carrier (PEG) were used to investigate the effect on bone formation of rhBMP-2 (Jung et al. 2008) and PTH (Jung et al. 2007a). Therefore, a comparison of these substances and those used in the present study can be performed. Compared to parathyroid hormone (PTH) (53.5 ± 22.7%) the mean area of newly formed bone was similar in the present study in the EMD 500/RGD group (54.8 ± 14.5%). The percentage of mineralized bone however (15.7 ± 5.2%) was lower than observed in the investigations with BMP (30.2 ± 7.6%) and PTH (19.6 ± 6%).

Based on the comparison of these 3 studies it seems that the effect on bone regeneration was similar between EMD and PTH, whereas rhBMP-2 revealed almost twice as much newly formed bone compared to either EMD or PTH. All 3 bioactive factors reveal a favorable in-growth and distribution of newly formed bone within the entire titanium cylinder. This might indicate a good matrix structure given by the PEG hydrogel.

Conclusion

It can be concluded that the combination of a polyethylene glycol (PEG) matrix containing enamel matrix protein (EMD) with HA/TCP granules has no effect on the percentage of mineralized bone tissue in the rabbit calvaria compared to spontaneous healing, independent of the EMD concentration. However, the addition of RGD peptide to the PEG/EMD500 group revealed an increased area fraction of new bone compared to the other treatment groups. Further studies are indicated to investigate a possible synergistic effect of EMD and RGD.

Acknowledgements

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Figures

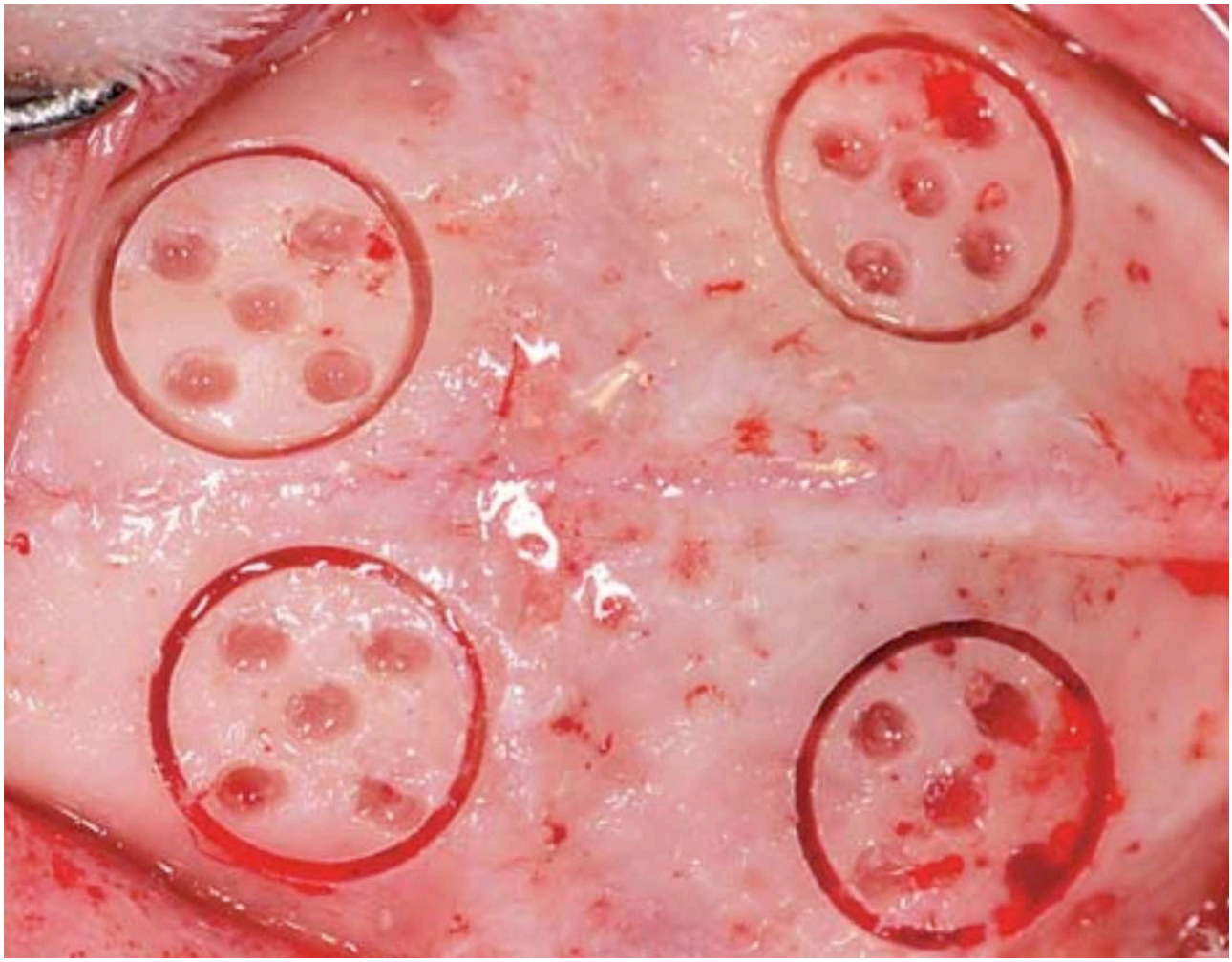

Figure 1: Bone preparation: Four circular slits (6mm in diameter, 1mm in depth) and five cortical perforation holes.

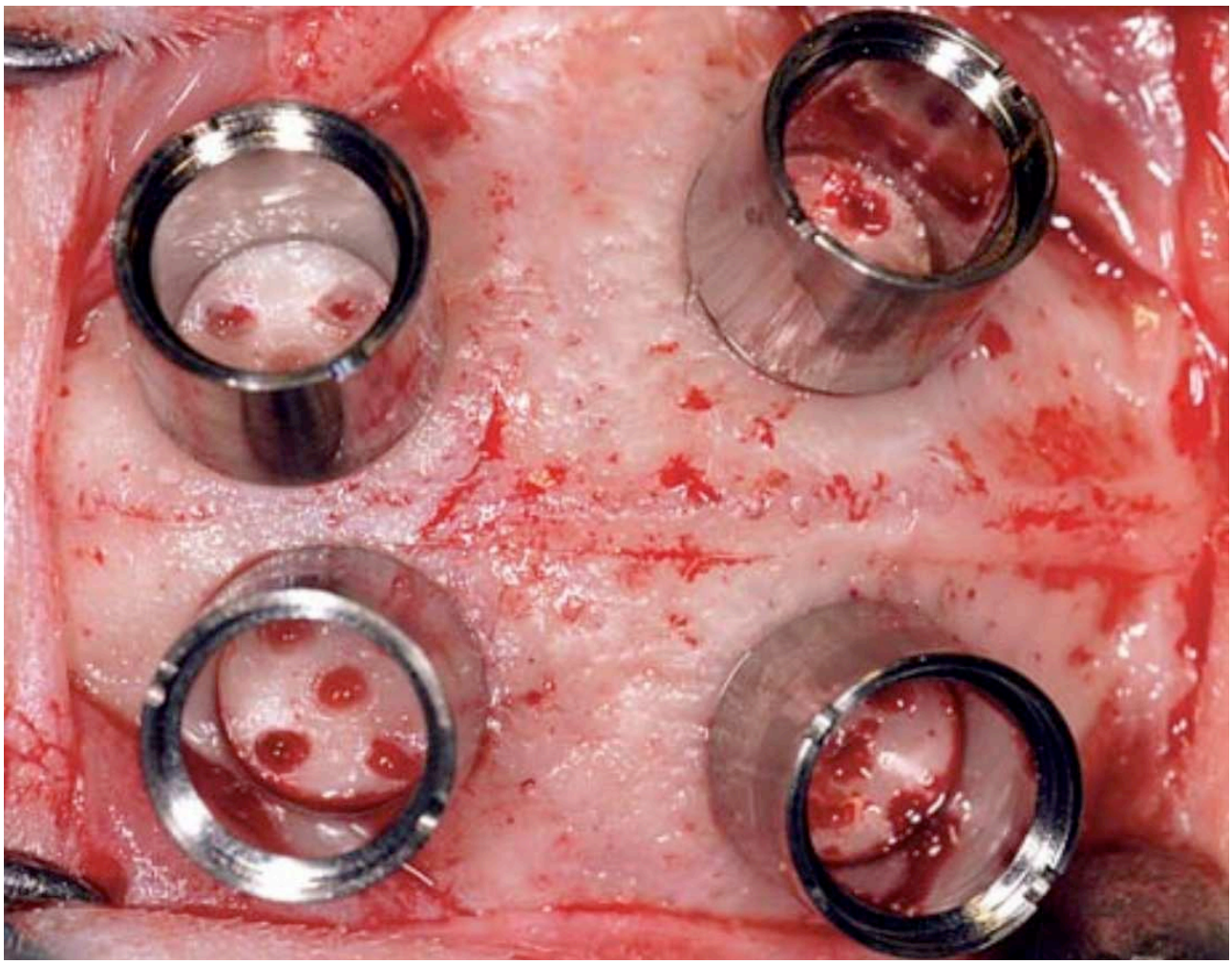

Figure 2: Titanium cylinders screwed into the slits.

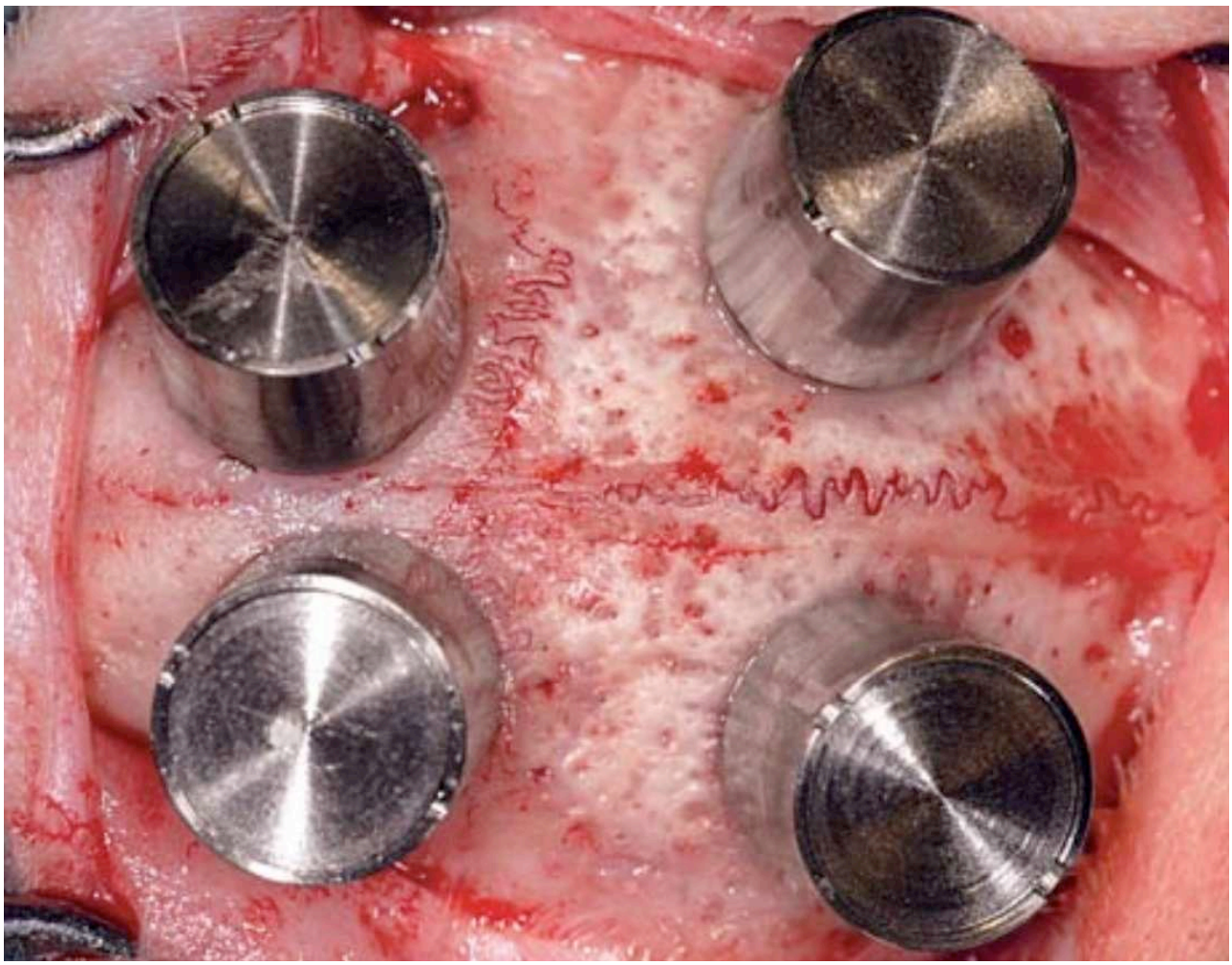

Figure 3: Closed cylinders after augmentation procedure.

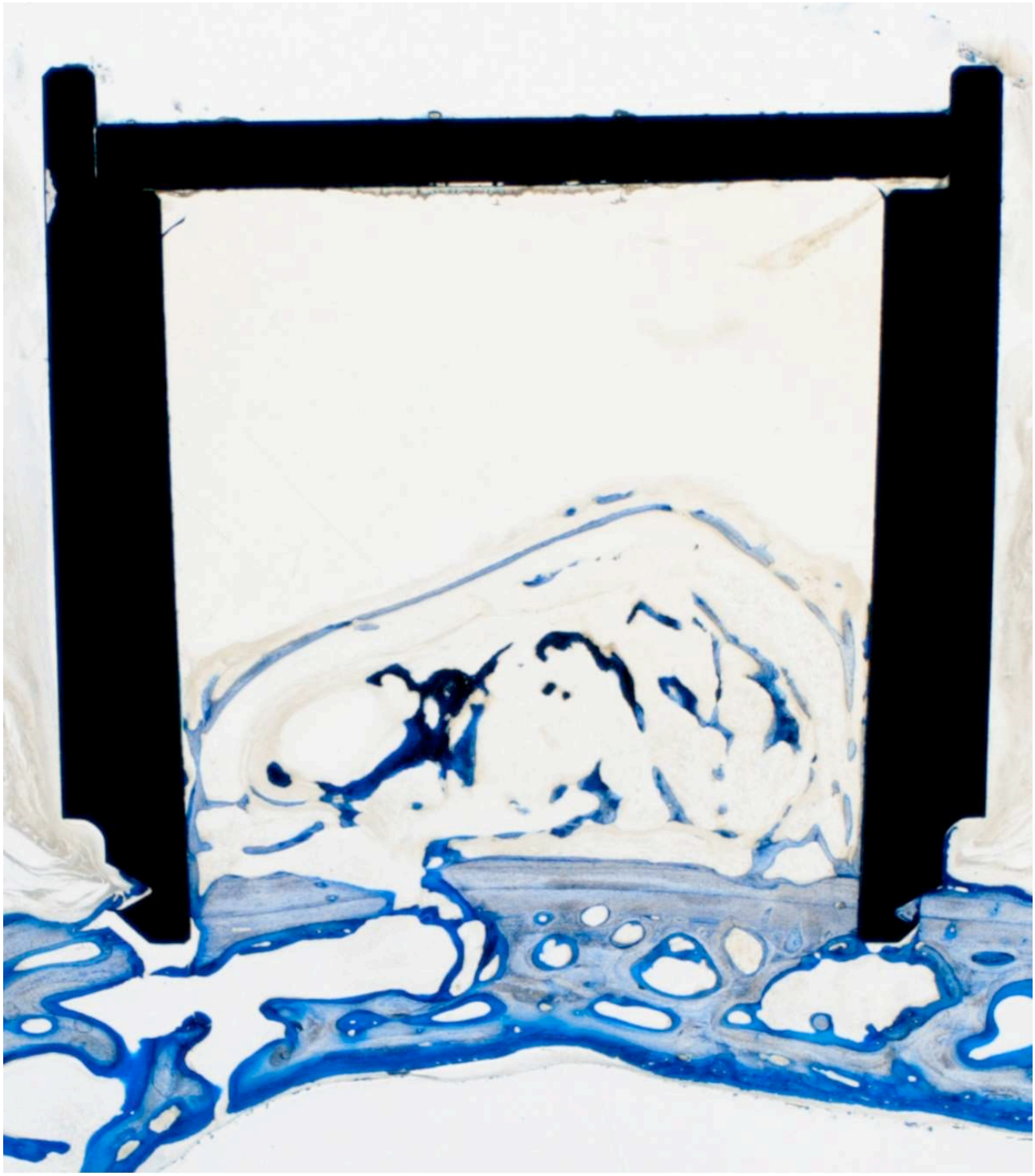

Figure 4: Histological section through initially empty cylinder (control group) (Original magnification x10).

Figure 5: Histological section through a cylinder containing PEG + EMD 100 (test 1) (Original magnification x10).

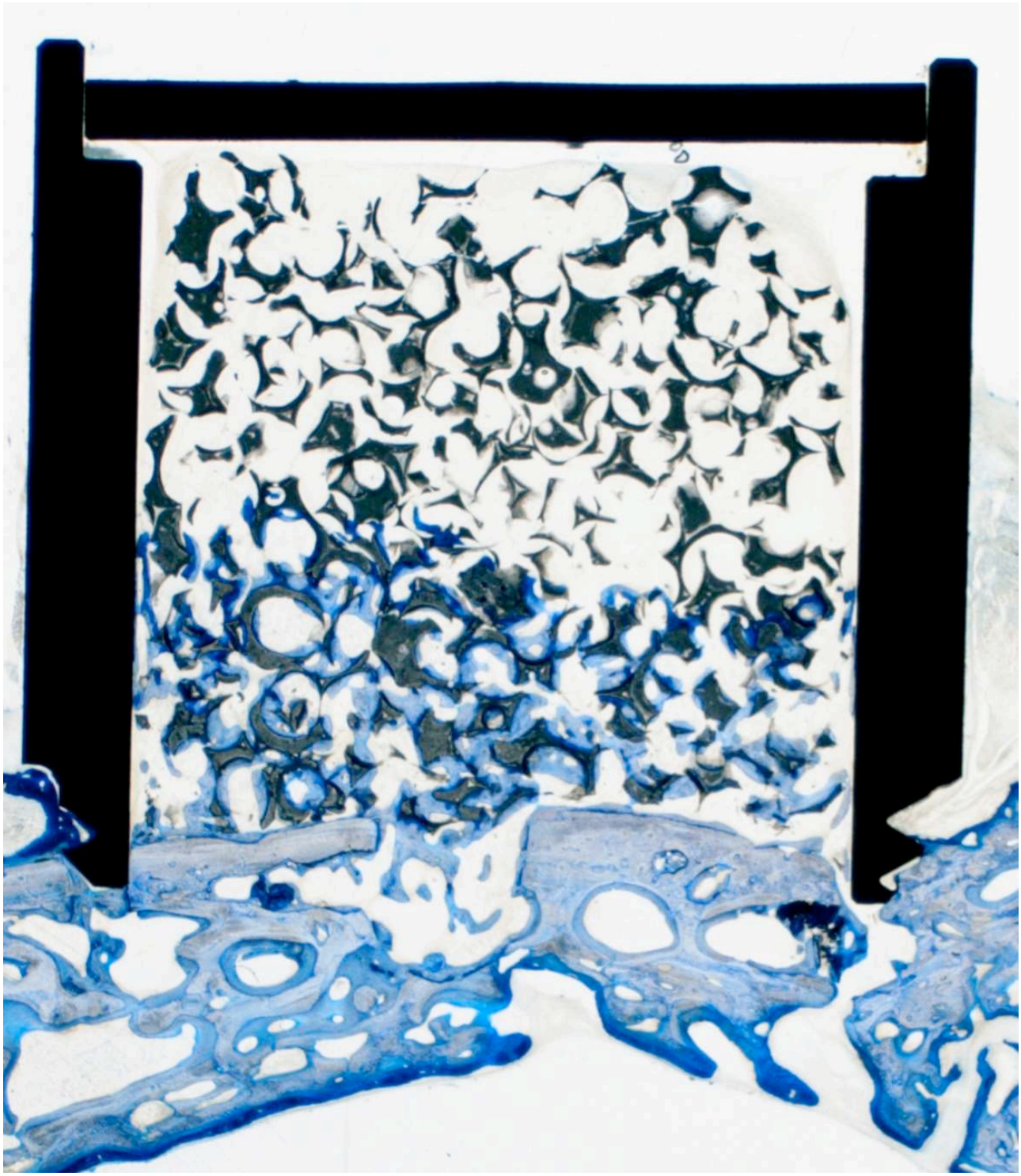

Figure 6: Histological section through a cylinder containing PEG + EMD 500 (test 2) (Original magnification x10).

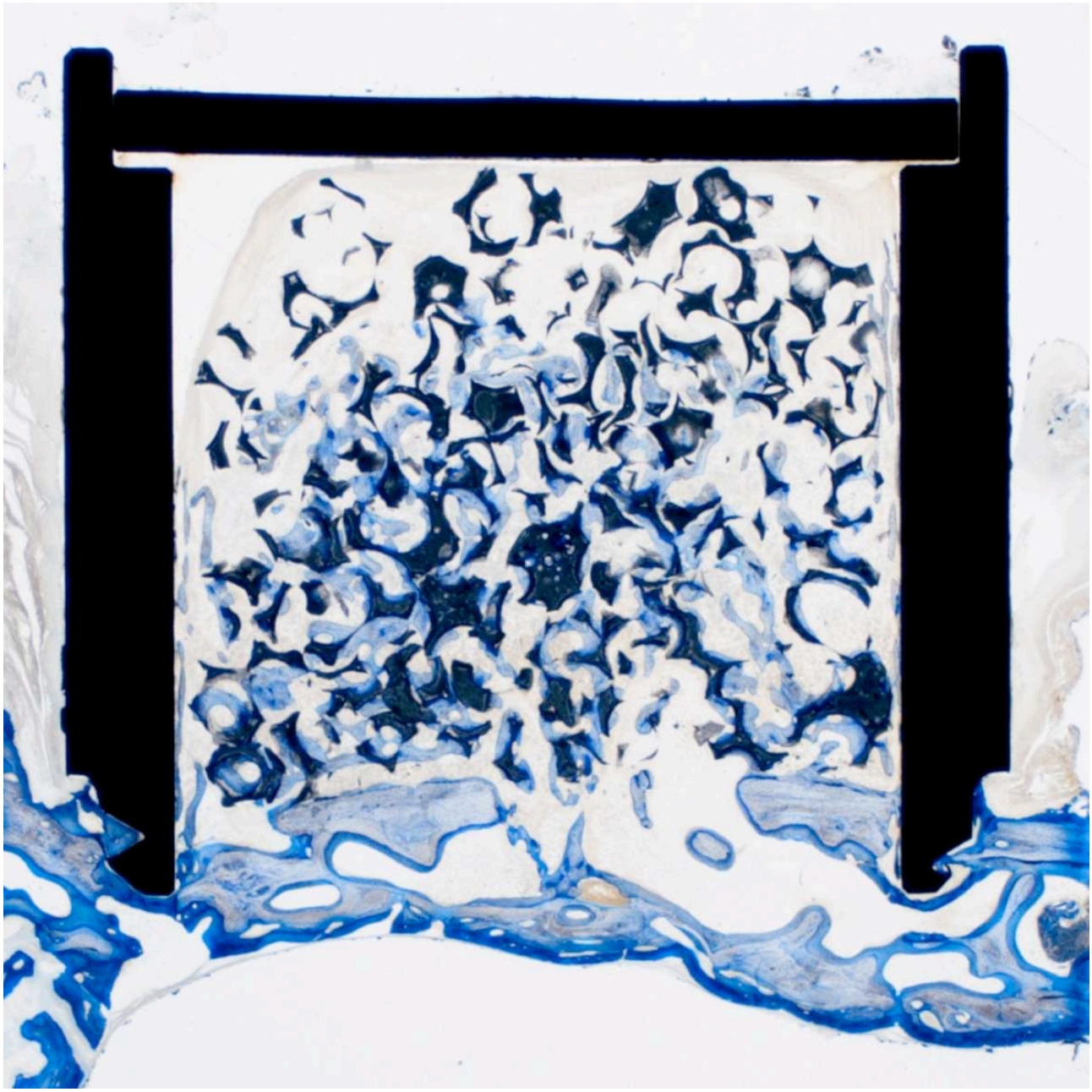

Figure 7: Histological section through a cylinder containing PEG + EMD 500 + RGD (test 3) (Original magnification x10).

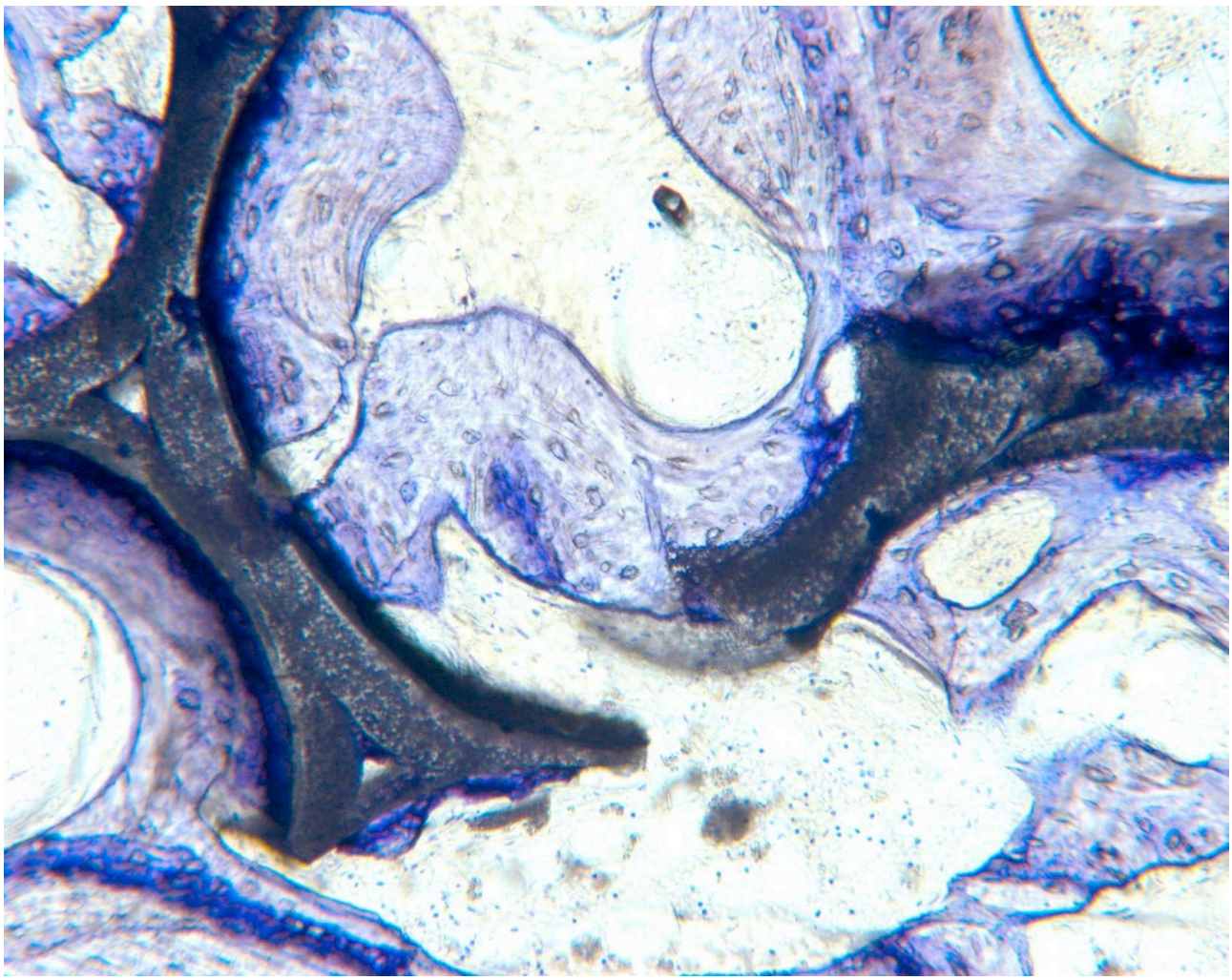

Figure 8: Histological section through a cylinder containing PEG + EMD 500 + RGD (test 3), detail (Original magnification x160).

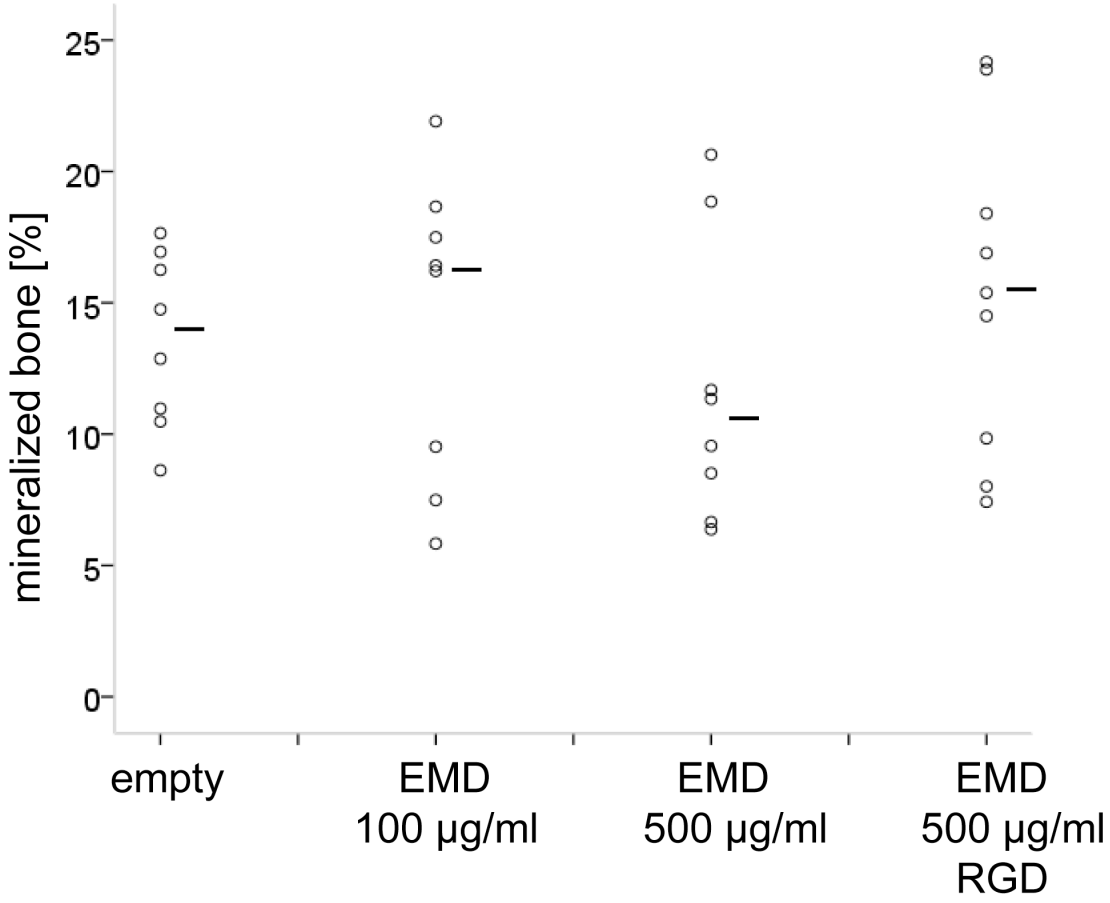

Figure 9: The percentage of mineralized bone generated within the cylinders in each group is displayed. The lines to the right of the values indicate the median of the group.. No significant differences between the groups were observed.

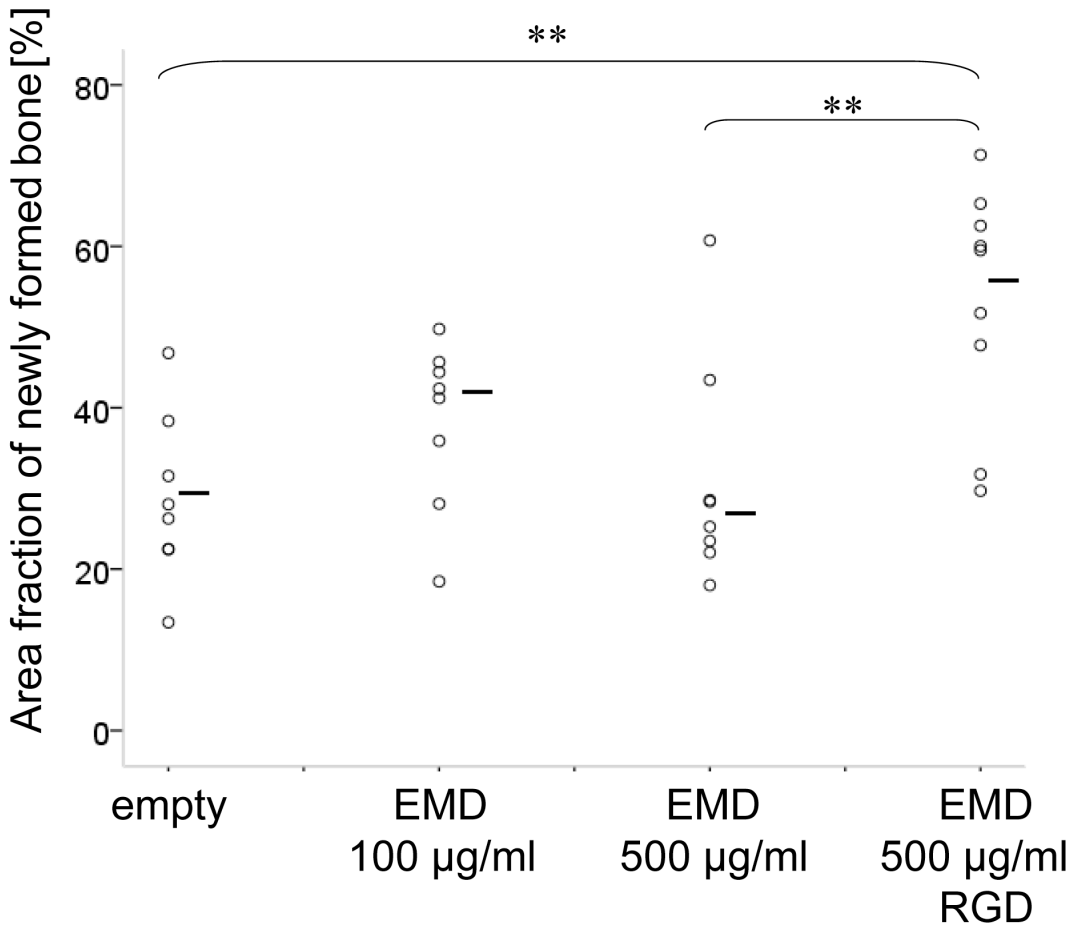

Figure 10: Area fraction of newly formed bone within the cylinder. The lines to the right of the values indicate the median of the group. Application of the Kruskan-Wallis test revealed a significant difference between the 4 groups (P=0.004).. Significant differences between groups determined by a Mann-Whitney U test are indicated ** P<0.05.

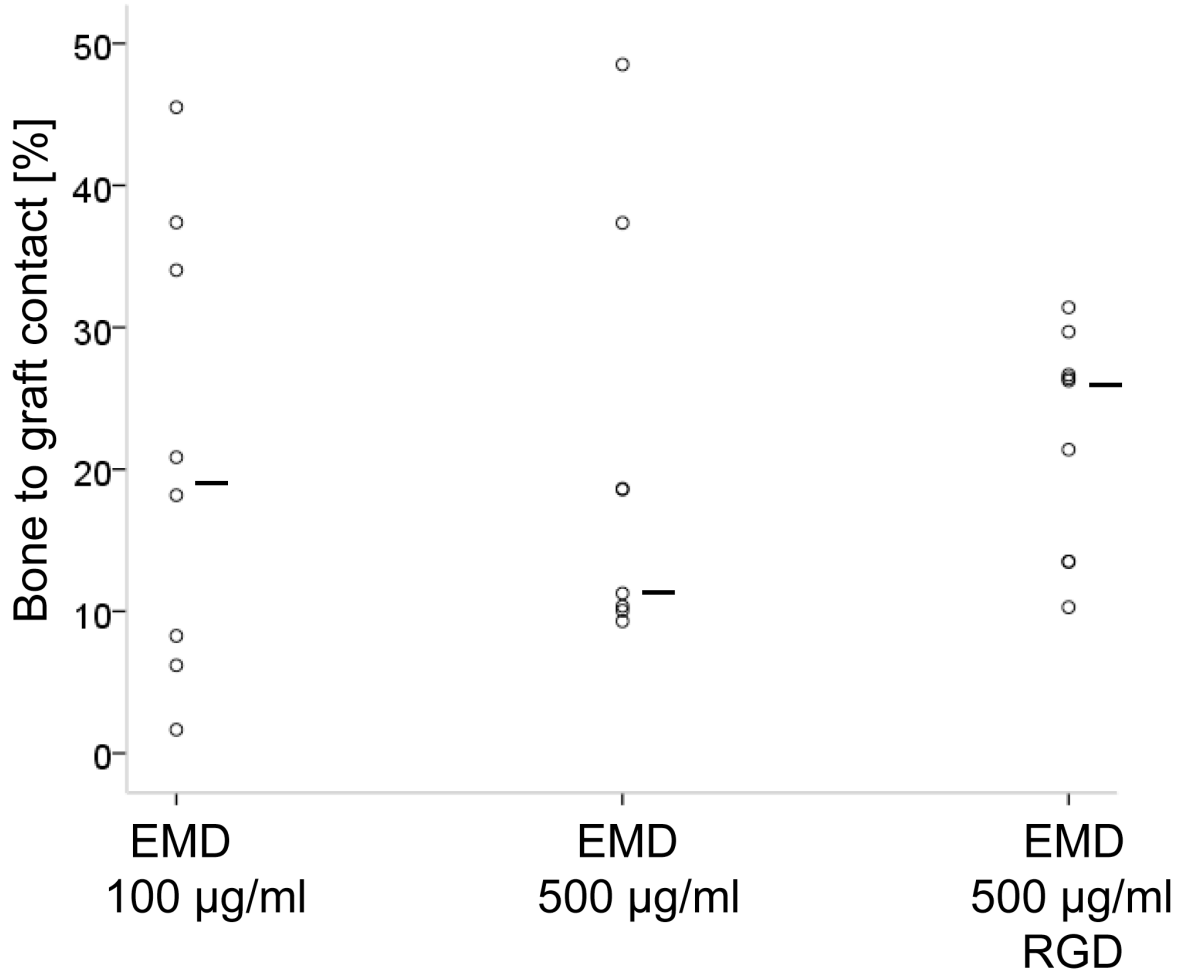

Figure 11: Surface fraction of bone to graft contact. The lines to the right of the values indicate the median of the group. No significant differences between the groups were observed.
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Percentage of mineralized bone			
Condition	Number of samples	Mean %	SD
Empty	8	13.6	3.3
EMD 100	8	14.2	5.8
EMD 500	8	11.7	5.9
EMD 500/RGD	9	15.7	5.2

Table 1: Percentage of mineralized bone

Area fraction of newly formed bone			
Condition	Number of samples	Mean %	SD
Empty	8	28.7	10.3
EMD 100	8	38.2	10.4
EMD 500	8	31.2	14.1
EMD 500/RGD	9	54.8	14.5

Table 2: Area fraction of newly formed bone

Surface fraction of bone to graft contact			
Condition	Number of samples	Mean %	SD
EMD 100	8	23.0	15.7
EMD 500	8	22.2	14.6
EMD 500/RGD	9	21.6	8.8

Table 3: Surface fraction of bone to graft contact